Conformational analysis of potent and very selective δ opioid dipeptide antagonists

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Abstract The δ selectivity and antagonism of peptides containing L-tetrahydro-3-isoquinoline carboxylic acid (Tic) in second position can be attributed mainly to the Tyr-Tic unit. These properties can be further enhanced by substituting Tyr[1] with 2,6-dimethyl-L-tyrosyl (Dmt). Dmt-Tic-NH_2, Dmt-Tic-OH, Dmt-Tic-Ala-NH_2 and Dmt-Tic-Ala-OH are all more active and/or selective than the corresponding [Tyr[1]]-parent peptides. In fact the selectivities of Dmt-Tic-OH and Dmt-Tic-Ala-OH are the highest ever recorded for opioid molecules. ^1H NMR spectra in a DMSO/water mixture at 278 K reveal the presence of two similar conformers, characterised by a *cis* or *trans* Dmt-Tic bond, in all four peptides. A detailed conformational analysis in solution of Dmt-Tic-NH_2 shows that these conformers have a shape very similar to that of the bioactive conformation of Tyr-Tic-NH_2 and to that of naltrindole.

Key words: Opioid dipeptide; Selectivity; Antagonism; Conformation; Nuclear magnetic resonance

1. Introduction

Several peptides containing L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (commonly dubbed Tic) in the second position behave as δ-selective opioid antagonists [1–4], whereas those beginning with the amino-terminal Tyr-*D-Tic*-Phe- unit are μ agonists, as expected [1]. For instance, enkephalin (a non-selective agonist) and dermorphin (a μ selective agonist) are both converted into δ selective antagonists [2] when their second residue is substituted with Tic. The surprising change of selectivity induced by the change of chirality in peptides containing the tetrahydro-3-isoquinoline carboxylic acid (Tic) in second position, originally interpreted [1] as a conformational preference induced on the Tyr-Xaa-Phe domain, can be attributed to the Tyr-Tic segment. In our view, this is an indication that the N-terminal Tyr-Tic sequence behaves as an effective recognition site, i.e. a sort of 'message domain' [5,6] for δ selective antagonism. In fact, even the simple Tyr-Tic-NH_2 and Tyr-Tic-OH dipeptides behave as a δ selective antagonists [4]. This interpretation has been recently confirmed by an independent study on tri- and tetra-peptides containing the amino-terminal Tyr-Tic- unit [7]. On the other hand, our di- and tri-peptides, while endowed of δ selectivities comparable to those of TIP (Tyr-Tic-Phe-NH_2) and TIPP (Tyr-Tic-Phe-Phe-NH_2), have substantially lower affinity and antagonist potencies than TIP and TIPP [1]; so that it is difficult to determine whether the major contribution to antagonism comes from a Tyr-Tic- recognition site or from the well known Tyr-Xaa-Phe-message domain.

In order to clarify this issue we have synthesised peptides lacking either Phe[3] or Phe[4] but endowed with greater antagonist activity and/or δ selectivity than TIP or TIPP. The decisive lead in the design of these new peptides was furnished by the increase of activity [8–11] shown by peptides in which Tyr[1] is substituted by 2,6-dimethyl tyrosine (Dmt). The [Dmt[1]]-analogues of the quoted di- and tri-peptide antagonists [4] did prove extremely δ selective antagonists [12]. Here we present a detailed conformational analysis in solution of Dmt-Tic-NH_2, in comparison with the conformational state of Dmt-Tic-OH, Dmt-Tic-Ala-NH_2 and Dmt-Tic-Ala-OH.

2. Materials and methods

Syntheses and biological assays were performed as described in [12]. Energy calculations were based on the all atoms parametrization of the AMBER force field (as implemented in the SYBYL package) [13,14].

NMR measurements. NMR samples were prepared by dissolving appropriate amounts of each peptide in 0.5 ml of 90/10 (v:v) $DMSO_{d6}$/H_2O cryoprotective mixture to make 2 mM solutions. NMR spectra were run at 400 MHz on a Bruker AM-400 instrument equipped with an Aspect 3000 computer and at 500 MHz on a Bruker AMX-500 instrument equipped with an X-32 computer. DQF-COSY [15], TOCSY [16], NOESY [17] and ROESY [18] experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by time-proportional phase incrementation of the initial pulse [19]. NOESY spectra were run at different mixing times (75, 150, 200 and 300 ms) to check the possible presence of spin diffusion and to better calibrate the interproton distances extracted from cross-peak volumes. Distance constraints were derived from the NOESY spectrum recorded with a 75 ms mixing time, using the method of Esposito and Pastore [20] after correction for spin diffusion according to the method of Majumdar and Hosur [21]. Relative scaling of the constraints was achieved by using a value of 1.79 Å for the distance between the β geminal protons 4 and 4′ of the Tic side chain.

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Abbreviations: $DMSO_{d6}$, perdeuterated dimethylsulfoxide; Dmt, 2,6-dimethyl-L-tyrosyl; DQF-COSY, double-quantum filtered correlation spectroscopy; GPI, guinea pig ileum; MVD, mouse vas deferens; MeNTI, methylnaltrindole; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ROESY, rotating frame nuclear Overhauser effect spectroscopy; Tic, L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP, Tyr-Tic-Phe-NH_2; TIPP, Tyr-Tic-Phe-Phe-NH_2; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.

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Table 1
Binding properties and pharmacological characterization of peptides containing the Tyr-Tic[a] and Dmt-Tic[b] message domains

	$K_i\ \mu$ (nM)	$K_i\ \delta$ (nM)	$K_i\ \mu/K_i\ \delta$	GPI IC_{50} (nM)	MVD IC_{50} (nM)	pA_2
Tyr-L-Tic-NH_2	28,700	170	170	$>10^4$	$>10^4$ (ant)	6.0
Dmt-L-Tic-NH_2	**280**	**1.22**	**230**			**7.2**
Tyr-L-Tic-OH	28,400	190	150	$>10^4$	$>10^4$ (ant)	–
Dmt-L-Tic-OH	**3,300**	**0.022**	**150,000**			**8.2**
Tyr-L-Tic-Ala-NH_2	33,800	55	610	$>10^4$	$>10^4$ (ant)	6.2
Dmt-L-Tic-Ala-NH_2	**47**	**0.24**	**195**			**8.5**
Tyr-L-Tic-Ala-OH	8,300	56	150	$>10^4$	$>10^4$ (ant)	7.0
Dmt-L-Tic-Ala-OH	**5,800**	**0.28**	**21,000**			**8.4**
TIP[c]			141			7.9
TIPP[c]			1410			8.5
Naltrindole[c]			18			9.2

[a]Data from [4].
[b]Data from [12].
[c]Data adapted from [1].

3. Results and discussion

Table 1 summarises the pharmacological properties of Dmt-Tic-NH_2, Dmt-Tic-OH, Dmt-Tic-Ala-NH_2 and Dmt-Tic-Ala-OH together with those of the corresponding peptides containing the Tyr-Tic 'message' [4]. The affinities for both μ and δ receptors of [Dmt[1]]-peptides are in all cases significantly higher than those of the corresponding [Tyr[1]]-peptides. δ selectivity is only slightly changed for the two amides, as expected from literature data on other [Dmt[1]]-peptides [7–10], but increases enormously for the two peptides with a free carboxyl C-terminal. It is worth emphasising that although the pA_2's of Dmt-Tic-OH (8.2) and Dmt-Tic-Ala-OH (8.4) are comparable to those of TIPP (8.5) and naltrindole (9.2), their μ/δ selectivities

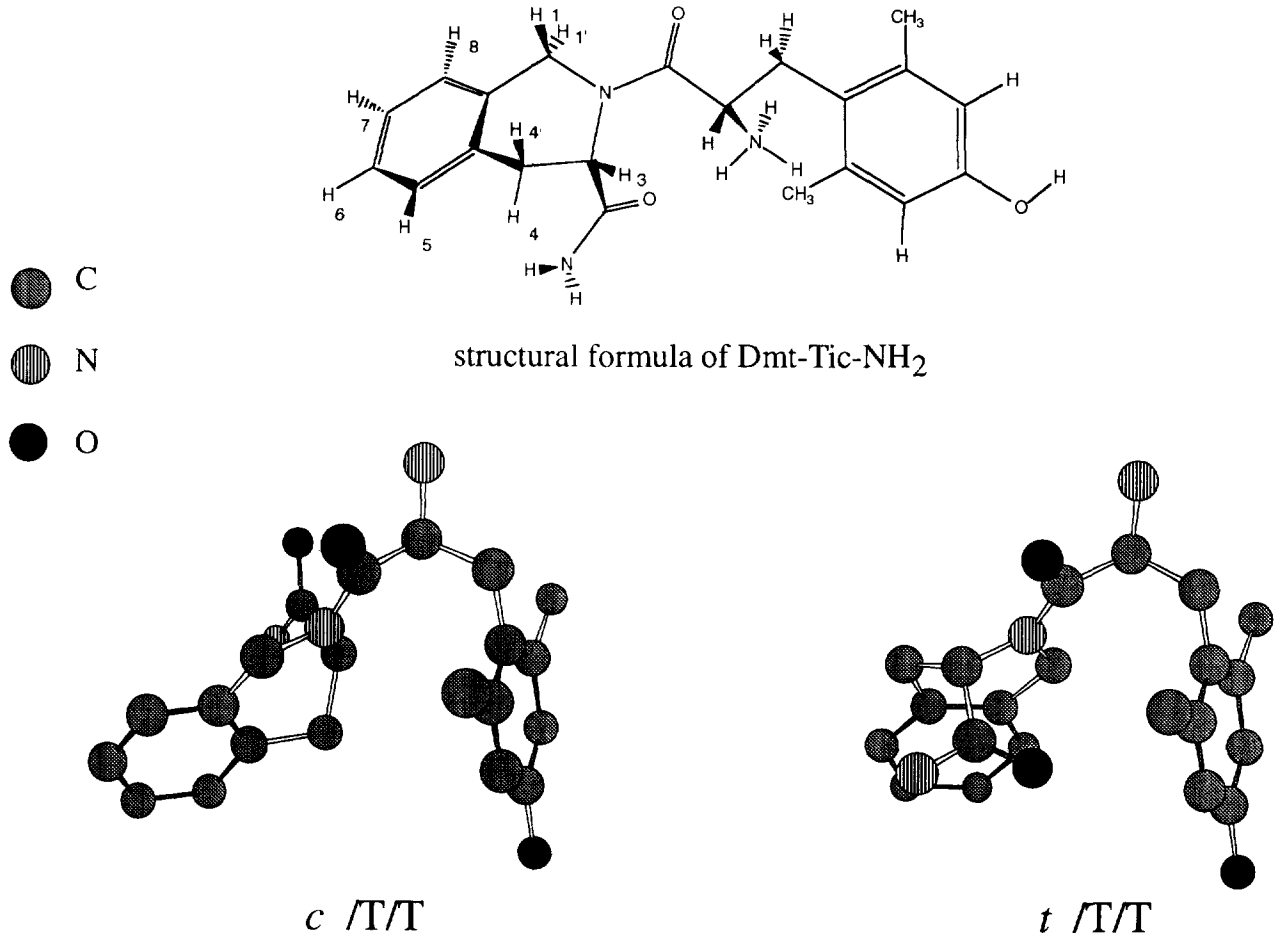

Fig. 1. Schematic formula of Dmt-L-Tic-NH_2 with numbering of the protons of the Tic side chain (top). Molecular models of the two conformers of Dmt-Tic-NH_2 observed in solution (*c*/T/T and *t*/T/T; bottom).

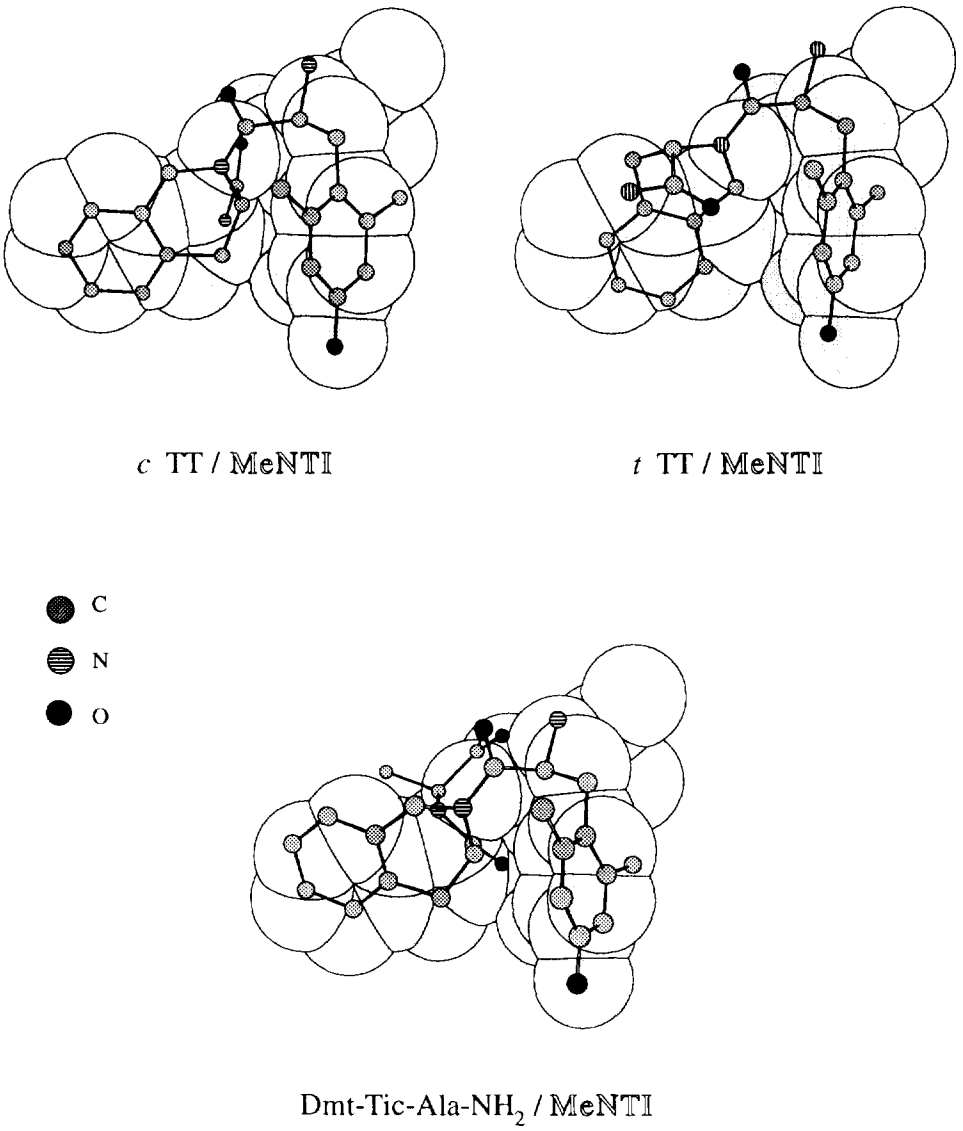

Fig. 2. Best fit of the two prevailing conformers of Dmt-Tic-NH_2 (*c*/T/T and *t*/T/T) and of the minimum energy*cis* conformer of Dmt-Tic-Ala-NH_2 with MeNTI; MeNTI is shown as space-filling model.

are one to four orders of magnitude higher (150,000 and 21,000 vs. 1410 and 18, respectively). The increase of selectivity found for Dmt-Tic-OH and Dmt-Tic-Ala-OH with respect to Dmt-Tic-NH_2 and Dmt-Tic-Ala-NH_2 is consistent with Schwyzer's hypothesis on the role of charges [5,6] for receptor selection but is so large that it may reflect conformational changes or specific receptor interactions as well.

In order to investigate the role of conformation in determining the affinities and selectivities of Table 1 we undertook a ^1H NMR study in solution. The ^1H NMR spectra of all four [Dmt^1]-peptides, in DMSO or in a 90/10 (v:v) $DMSO_{d6}/H_2O$ cryoprotective mixture, are characterised by the presence of two stable conformers which have similar NMR parameters for the two initial residues common to all peptides, indicating that the conformations, in each peptide, are dominated by the conformational preferences of the Dmt-Tic moiety. Accordingly we chose to investigate the conformations of Dmt-Tic-NH_2 in greater detail since, on the basis of the NMR data, they are also representative of the corresponding conformers of the other peptides.

NOESY and ROESY spectra of Dmt-Tic-NH_2 in $DMSO_{d6}$ at room temperature show a very large number of cross-peaks for a peptide of this size. Notwithstanding, to make a homogeneous comparison with the solution study of Tyr-Tic-NH_2, we ran all quantitative experiments in a 90/10 (v:v) $DMSO_{d6}/H_2O$ cryoprotective mixture [2] at 278 K. In fact, we have shown that the use of biocompatible media with viscosities of the order of 7–10 cp, not only leads to a quantitative increase of all NOEs observed at lower viscosity, as expected by the theory of microviscosity, but also to selective growth of (conformationally diagnostic) effects involving backbone protons with respect to intrachain ones [22].

The NOESY spectrum of Dmt-Tic-NH_2 in the 90/10 (v:v) $DMSO_{d6}/H_2O$ cryoprotective mixture at 278 K contains two subspectra corresponding to two conformers, or families of conformers, arising from the *cis/trans* isomerism around the

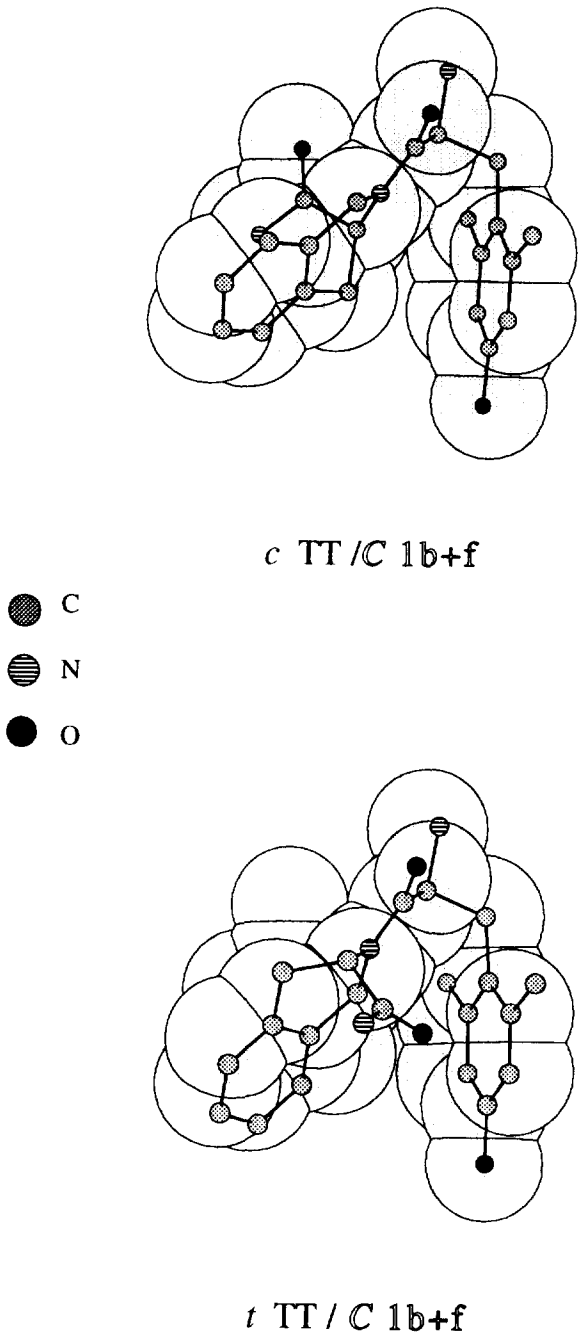

Fig. 3. Best fit of the two prevailing conformers of Dmt-Tic-NH_2 (*c*/T/T and *t*/T/T) with the model of conformer C1b + f of Tyr-L-Tic-NH_2. C1b + f is shown as space-filling model.

Dmt-Tic bond. The relative populations of the two conformers A and B are 0.75 and 0.25, respectively. The assignment of A and B to *cis*/*trans* families was made through diagnostic NOEs: the C_α-C_α effect characteristic of a *cis* Dmt-Tic bond was observed in the subspectrum of A, whereas that of B shows a cross peak between the Dmt C_α and the Tic 1, 1′ protons characteristic of a *trans* Dmt-Tic bond (see Fig. 1 for numbering).

Isomers A and B can differ in the conformations of the two side chains but their identification is simplified by the experimental observation that the aliphatic moiety of the Tic ring has the same conformation in both isomers. In fact, the $J_{\alpha,\beta}/J_{\alpha,\beta'}$ coupling constants have pairs of values for all isomers that are only consistent with a half-chair conformation very similar to that observed for Tyr-Tic-NH_2 [2,4]: 3.5/5.3 and 3.8/5.9 for A and B, respectively (all measured in Hz). Thus, isomers A and B can only differ in ψ_1, χ_1 and χ_2 values.

In order to facilitate the interpretation of the NMR data we undertook an exhaustive conformational search of Dmt-Tic-NH_2 by holding the peptide bond *trans* for B and *cis* for A, the ring conformation of Tic fixed in a half-chair conformation and varying ψ_1, χ_1 and χ_2 systematically in steps of 15°. All minimum energy conformers have similar values of χ_2 (of the order of 90°); therefore, in the following, isomers will be symbolically designated by a *c* (for *cis*) or a *t* (for *trans*) followed by the values of ψ_1, χ_1 closest to 180° (*T*), 60° (*G*−) or −60° (*G*+).

This conformational analysis yields 14 low energy conformers within a range of 20 kcal/mol from the absolute energy minimum corresponding to conformer (*c*/T/T), six with a *cis* and eight with a *trans* peptide bond. Among the six *cis* conformers of relative minimum energy, only (*c*/T/T), the absolute minimum, is fully consistent with the NMR parameters of A. The NMR data that characterise A molecules are the pronounced upfield shifts of the resonances of the α, β and β' protons of Tic and the concomitant NOEs of Tic H_α with both 2 and 6 methyl groups and with Tic 1. It can be seen from the molecular model of Fig. 1 that the ring of Dmt can induce prominent ring-current shifts on the α, β and β' protons of Tic to account for their upfield resonances whereas the diagnostic NOEs between Tic H_α with methyl groups and with Tic 1 do correspond to short distances in the model. Similar considerations lead to the assignment of B to conformer (*t*/T/T) whose energy is 2.97 kcal/mol above that of (*c*/T/T). The spectra of the other peptides can be interpreted in terms of very similar conformers, as far as the Dmt-Tic moiety is concerned, with small quantitative differences due to the influence of the C-terminal part. The molecular models of the two minimum energy (*cis* and *trans*) conformers of Dmt-Tic-NH_2 are reported in Fig. 1 along with the scheme of the molecule indicating the numbering of the protons of the Tic side chain. In spite of the different configuration of the peptide bond, both conformers (i.e. *c*/T/T and *t*/T/T) have a similar compact shape reminiscent of that of the conformer of Tyr-Tic-NH_2 (C1b + f) that we proposed as the bioactive conformation of that antagonist [2,4].

These models can now be used for comparison with conformationally rigid δ selective opiates to test their consistency with the hypothetical bioactive conformation. There are several naltrindole derivatives with a fairly good δ selectivity, both agonists and antagonists. For consistency with previous work on δ antagonists [2,4] we selected once again N-methyl naltrindole (MeNTI) as a typical rigid δ antagonist [23]. Superpositions with the models of our peptides were generated by overlaying only a portion of the tyramine moiety of the peptides with the corresponding atoms of MeNTI: N, C_α, C_β and the C-1 and C-4 carbons of the aromatic ring. In fact, it must be noted that the ortho and meta carbon atoms of the aromatic ring of Tyr (or Dmt of any opioid peptide) cannot possibly overlap with the corresponding atoms of any alkaloid related to morphine since the orientation of the ring depending from the χ_2 torsion angle is forced by cyclization (in all alkaloids) to values close to 0° that are energetically inaccessible in linear molecules. It can be

seen from Fig. 2 that both conformers of Dmt-Tic-NH_2 are consistent with the shape of this rigid non-peptidic agonist. As anticipated in the presentation of the NMR data, the preferred conformations of the other [Dmt-Tic-] peptides are very similar to those found for Dmt-Tic-NH_2, i.e. (*c*/T/T) and (*t*/T/T). As an example, Fig. 2 shows also the fit of the minimum energy *cis* conformer of Dmt-Tic-Ala-NH_2 with MeNTI; it can be seen that the N-terminal Dmt-Tic- moiety has almost exactly the same fit as in conformer (*c*/T/T) of Dmt-Tic-NH_2.

Fig. 3 shows a comparison of conformers (*c*/T/T) and (*t*/T/T) with the molecular models of conformer C1b + fof Tyr-Tic-NH_2. It can be appreciated that they are very similar, in particular, all the corresponding atoms of (*c*/T/T) and C1b + f are coincident, with a RMS error of 0.18 Å in the overlay. These findings lead strong support to our proposal [2,4] that the 90° arrangement of the two aromatic rings of conformer C1b + f is consistent with specific δ receptor requirements. In fact, all the peptide models of Fig. 3 are consistent with the shape of the rigid δ selective antagonist MeNTI [4,23,24].

It is also noteworthy that both (*c*/T/T) and (*t*/T/T), which account for all conformers of Dmt-Tic-NH_2, are consistent with receptor requirements whereas, in the case of Tyr-Tic-NH_2, conformer C1b + f, the only low-energy conformer consistent with the shape of MeNTI, represented only 40% of all solution conformations. Yet, the increase of relative populations of bioactive conformers observed in solution is not sufficient per se to explain the high affinity and selectivity of the [Dmt^1]-peptides. The increased δ selectivity observed when the C-terminal amides are changed into carboxyl groups is consistent with the role of negative charges in the membrane-assisted receptor-selection [5,6] but is too large to be fully accounted for. It seems probable that the two methyl groups of Dmt, while favouring a better fit of the tyramine moiety in the T subsite [25,26] of both receptors, impose more severe constraints on the interaction of the whole molecule with its receptor. In other words, [Dmt^1]-peptides are only slightly more rigid in solution but much more rigid inside the receptors, e.g. the good fit of Dmt might force the carboxyl groups of Dmt-Tic-OH and Dmt-Tic-Ala-OH to come into close contact with the anionic site of μ receptors. It is somewhat surprising that our peptides are much more selective than naltrindole. Such a behaviour may reflect mutual adaptability of antagonists and receptors. That is, our (relatively flexible) peptides can fit only the δ receptor directly without the need for energetically difficult conformational transitions, whereas the rigid naltrindole may force the μ receptor to adapt to its shape.

At any rate it seems fair to conclude that the bioactive conformation is the same for [Tyr^1-Tic]-peptides and [Dmt^1-Tic]-peptides, with a recognition site ('message domain') confined to the first two residues. The presence, in [Tic^2]-peptide antagonists, of Phe^3 and/or Phe^4 [1,27] may further stabilise the bioactive conformation (of the first two residues) or introduce additional bioactive conformations (with Tyr-Tic-Phe- as 'message') but is by no means necessary to determine antagonism.

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